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(54) Title: LIPOSOMAL RADIOLOGIC CONTRAST AGENTS

(57) Abstract

Contrast agents for magnetic resonance imaging comprising liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or super-paramagnetic agent, and liposomes, however prepared, having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic and/or superparamagnetic agent are described, as well as methods for imaging the blood pool and for diagnosing the presence of tumorous tissue in a patient which utilize these contrast agents.

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TITLE

LIPOSOMAL RADIOLOGIC CONTRAST AGENTS

RELATED APPLICATION

This application is a continuation-in-part of pending application U.S.S.N. 269,190, filed November 9, 1988.

BACKGROUND OF THE INVENTION

Accurate detection of the presence, location and extent of splenic and hepatic tumors is of profound clinical significance. With recent advancements in oncological therapy, many malignant tumors can be effectively treated if their existence is promptly and correctly ascertained. Over the years, the medical profession has relied on various imaging methods in an effort to provide such diagnoses. Many of these imaging methods, however, have disadvantages and/or limitations associated with their use.

Radionuclide scans, for example, were used early on for the detection of malignancy within the liver and spleen. Such scans rely on the uptake of particulates, such as technetium sulphur colloid, by the Kupffer cells of the liver and spleen. Resolution, however, is poor, and

the use of this procedure for malignancy detection has been limited.

Computed tomography (CT), an imaging method that provides high spatial resolution, has been used extensively for the detection of liver and splenic malignancy. CT relies on the differential linear attenuation of tumors and adjacent normal hepatic or splenic tissue (parenchyma). Generally malignancies have a somewhat lower attenuation than adjacent parenchyma, and are thus detectable on CT. In some cases, however, tumors show similar attenuation as the parenchyma and, as such, are not visible upon non-contrast CT examination.

To improve the detection of liver and splenic tumors on CT, iodinated water soluble contrast agents have been employed. These iodinated compounds are distributed throughout the extracellular fluid and lead to the detection of approximately 16% additional malignancies. Such contrast agents, however, rely upon blood flow for delivery. Therefore, a hepatic or splenic tumor with similar blood flow as surrounding tissue often is not detected using contrast enhanced CT. Overall, only about 70% of hepatic metastases are detected using this method, and for malignant lymphomas, the detection rate is even poorer. Lymphomatous involvement of the spleen is also extremely difficult to detect on CT, with or without contrast media, this method having at best a sensitivity of only about 50% where splenic lymphoma is involved.

The disappointing level of improvement observed with the extracellular iodinated contrast media led to the search for tissue specific contrast agents for use in CT detection of hepatic and splenic tumors. Much of the effort focused on either oily contrast media such as ethiodol (EOE-13) or particulate contrast agents such as microencapsulated or liposomally encapsulated iodinated contrast media. These oily or particulate iodinated contrast agents are accumulated preferentially by normal cells of the liver, spleen and bone marrow, particularly by Kupffer cells. Clinical trials have shown that these agents improve CT detectability, especially where lymphoma identification is concerned. CT, however, requires a relatively high dose of contrast agent (in the amount of about 10 millimolar iodine) to provide differential contrast and, unfortunately, such contrast agents have proven toxic at this dosage. Thus, while helpful in improving contrast, problems such as toxicity have restricted the use of CT employing these tissue specific contrast agents to certain centers and experimental trials.

Magnetic resonance imaging (MRI) has proven to be the most useful detection technique for identifying hepatic and splenic malignancies. In MRI, tumors are detected because of a difference between the relaxation times (T_1 and/or T_2) of normal and malignant tissue. This difference in T_1 and/or T_2 causes differential signal intensity (contrast) on MR images, with greater differences in T_1 (or T_2), leading to greater contrast. Typically, liver tumors

have a longer T_1 and a longer T_2 than corresponding normal tissue. This results in tumors showing a hypointensity on T_1-weighted MR images and a hyperintensity on T_2-weighted MR images, relative to surrounding parenchyma. In some instances, however, malignancies and normal tissue may be isointense on MRI, and therefore nondetectable.

In an attempt to lower the instances of isointensity and increase the overall detection rate of this imaging method, agents capable of enhancing tissue and tumor contrast were sought. The major MRI agent developed as a result of this effort was the paramagnetic compound gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA), discussed in Weinmann et al., A.J.R., Vol. 142, pp. 619-624 (1985); Brasch et al., A.J.R., Vol. 142, pp. 625-630 (1984); and Strich et al., Radiology, Vol. 154, pp. 723-726 (1985). Under certain conditions, free Gd-DTPA is capable of providing good contrast enhancement in the micromolar range, approximately two orders of magnitude lower in concentration than required for iodinated contrast enhancement on CT. When effectively employed, Gd-DTPA results in concentration dependent decreases in either normal tissue T_1 and T_2 or tumor T_1 and T_2, but not both. The higher the concentration of contrast agent that reaches the tissue, the greater the measured effect on T_1 and T_2. The measured T_1 (or T_2) is given by the formula

$$1/T_1 \text{ (measured)} = 1/T_1 \text{ (tissue)} + 1/T_1 \text{ (contrast)}$$

where $1/T_1$ (tissue) is the T_1 in tissue in the absence of contrast agent and $1/T_1$ (contrast) is the contribution to tissue T_1 caused by the contrast agent. As the foregoing formula shows, the relaxation rates are additive. Gadian et al., A.J.R., Vol. 143, pp. 215-224 (1985); and Brown et al., Med. Phys., Vol. 11, pp. 67-72 (1984).

Unfortunately, human trials with free Gd-DTPA evaluating its use in diagnoses involving the liver and spleen have been disappointing. One problem is that free Gd-DTPA, like the iodinated contrast media employed in CT, is an extracellular agent which is distributed throughout the extracellular fluid space proportional to blood flow. Because the relaxation rates are additive, the effect of contrast agent is proportionately greater on tissues with longer relaxation times than for tissues with shorter relaxation times. Tumors tend to exhibit longer relaxation times than normal tissue. Damadian et al., Science, Vol. 171, pp. 1151-1153 (1971). Accordingly, if the contrast agent equilibrates between tumor and normal tissue, the effect will be to decrease the tumor T_1 (and T_2) more than the normal tissue T_1 (and T_2), which will result in a decrease in the contrast between the tissues. Thus, where tumors have similar blood flow as surrounding tissue, the tumor T_1 (and T_2) is also affected, and such tumors often go undetected.

Research into increasing the effectiveness of Gd-DTPA as an MRI contrast agent has focused on the use of liposomes to encapsulate and selectively deliver Gd-DTPA to

liver and splenic parenchyma. Liposomes are cleared principally by the liver, spleen and bone marrow, and thus should be accumulated preferentially by such tissues. The primary collecting cells within these tissues are the phagocytic Kupffer cells. Tumors, however, generally do not contain such cells. Thus, theoretically, liposomes can be employed to selectively target contrast agents to normal hepatic, splenic and bone marrow tissue, without regard to blood flow, thereby increasing the contrast between the liver, spleen or bone marrow and any tumor cells.

A number of methods are available in the art to produce liposomes. For example, unilamellar lipid vesicles can be prepared from organic solvents by reverse phase techniques which involve either solvent dilution (Deamer et al., Biochim. Biophys. Acta, Vol. 443, pp. 629-634 (1976); and Kremer et al., Biochemistry, Vol. 16, pp. 3932-3935 (1977)) or solvent evaporation (Szoka et al., Proc. Natl. Acad. Sci., Vol. 79, pp. 4194-4198 (1978)). These procedures suffer from the common limitation that not all lipids are equally soluble in all organic solvents, thus the solvents employed must be varied when the lipid composition is altered (Schieren et al., Biochim. Biophys. Acta, Vol 542, pp. 137-153, 1978); and Deamer in Liposome Technology, Vol. 1 (CRC Press 1984) (Greguriadis, G., ed.)). Additionally, there is a need to remove residual solvents from the lipid preparations by chromatographic or dialysis techniques. The vesicles produced by such methods tend to be a heterogenous mixture of multilamellar and

unilamellar vesicles. Thus, it is necessary to size the vesicles using various techniques such as by extrusion through polycarbonate filters. Olson et al., Biochim. Biophys. Acta, Vol. 557, pp. 9-23 (1978). U.S. Patent No. 4,752,425 discloses liposomes produced by such solvent dilution and extrusion techniques. In addition, U.S. Patent No. 4,737,323 describes a size-processing method for heterogeneous liposome suspensions prepared from various techniques. The processing described involves passing a suspension of the heterogeneously sized liposomes, wherein the liposome sizes are predominantly greater than about one micron, through an asymmetric ceramic filter having an inner surface pore size of about one micron.

Detergent removal represents another general method of liposome vesicle formation. In such methods, either the lipid materials or the pre-formed vesicles are solubilized in detergent to form mixed micelles. The detergent is then removed by dialysis and the micelles coalesce to form bilayer vesicles. One problem with this technique is that the particular dialysis method which can be effectively employed is heavily dependent upon the specific lipids being used. For example, different lipids may require the use of different lipid to detergent ratios (Mimms et al., Biochemistry, Vol. 20, pp. 833-840 (1981); Enoch et al., Proc. Natl. Acad. Sci., Vol. 76, pp. 145-149 (1979); and Weder et al., in Liposome Technology, Vol. 1, (CRC Press 1984) (Gregoriadis, G., ed.)) and the rate of detergent removal by dialysis or by gel filtration may need

to be changed in order to obtain reproducible preparations (Mimms et al., Biochemistry, Vol. 20, pp. 833-840 (1981); and Schuh et al., Biochim. Biophys. Acta, Vol. 687, pp. 219-225 (1982)). Another major problem with this technique is the difficulty in removing all the residual detergent (Madden, Chem. Phys. Lipids, Vol. 40, pp. 207-221 (1986)).

Alternatively, unilamellar lipid vesicles may be prepared from multilamellar vesicles (or frozen and thawed multilamellar vesicles) by mechanical techniques involving cavitation and shearing (french press) (Lelkes, in Liposome Technology, Vol. 1, pp. 51-65 (CRC Press 1984) (Greguriadis, G., ed.)), sonication (Mayhew et al., in Liposome Technology, Vol. 2, pp. 19-31 (CRC Press 1984) (Greguriadis, G.)), or filter extrusion (Mayer et al., Biochim. Biophys. Acta., Vol. 858, pp. 161-168 (1986)) methods.

As those familiar with the art would recognize, the aforementioned sonication method has several limitations. First, it is difficult to make sonication a completely reproducible procedure. This is due in part to the fact that sonication involves the transfer of energy from the transducer to the medium to be sonicated. The efficiency of energy transfer between the transducer and material to be sonicated depends upon many factors. These include the concentration of the lipid dispersion to be sonicated, the ionic strength of the medium in which the lipid is dispersed, whether a probe or bath-type sonicator is used, as well as other factors. In the case of

sonication involving physical contact between the probe tip of the sonicator device and the material to be sonicated, the physical positioning of the probe tip within the dispersion can influence the sonication process. In cases where the medium to be sonicated is physically separate from the energy transducer of the sonicator, e.g., in a bath-type sonicator, the energy transfer will be modulated by the geometry and wall thickness of the vessel containing the material to be sonicated. It is also well recognized that it is difficult to employ the sonication process at lipid concentrations above about 100 mg/ml and achieve uniform size distributions. Since lipid concentrations are low, the trapping efficiency for any water-soluble solute will also tend to be low. Another disadvantage is that it is necessary to sonicate for long times in order to achieve uniform size distributions. Furthermore, in the process of sonication, the energy applied to the sonicate is liberated partially as heat. It is therefore necessary to cool the sample under sonication to avoid thermal degradation of the labile lipids. Moreover, it is also a difficult procedure to scale up. When using probe-type sonicators, for example, there is the possibility of contamination of sonicate with metallic particles from the tip of the probe. This must either be tested for or else the sonicate centrifuged to remove such particles from the sonicate, thus complicating the preparatory procedure further. Lastly, it has been reported that lipid vesicles prepared by sonication are unstable and tend to fuse (Parente et

al., Biochemistry, Vol. 23, pp. 2353-2362 (1984)). This is undesirable since it is known that the overall size of lipid vesicles influences their biodistribution (Cullis et al., in Liposomes, pp. 39-72 (Marcel Dekker 1983) (Ostro, M., ed.)).

The french press method noted above involves the extrusion of lipid through a small orifice under high (approx. 20,000 psi) pressure. The procedure is rapid and versatile, but is, however, limited to relatively low lipid concentrations (< 50 mg/ml) which limits the trapping efficiency of the technique.

Related to the aforementioned french press method is the process of extruding lipid dispersions through filters of defined pore size. Mayer et al., Biochim. Biophys. Acta, Vol. 858, pp. 161-168 (1986). International Application PCT/US85/01161 describes such extrusion techniques as suitable for preparing liposomes of various lipid combinations having a substantially unimodal size distribution and a unilamellar construction. The unimodal size distribution results from repeatedly passing previously prepared liposomes through one or more filters each having the same pore size. The unilamellar construction is achieved by employing a filter having a pore size of about 100 nm or less. The preparation of liposomes directly from lipid pellets or powder and buffer, thus avoiding the addition of any extraneous materials such as solvents, detergents, etc., and the use of freeze-thaw cycles prior to extrusion to increase the trapped volumes

of the liposomes, are also discussed. The liposomes thus prepared are described as useful in delivering entrapped material in vivo. Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985) and Mayer et al., Biochimica et Biophysica Acta, Vol. 817, pp. 193-196 (1985) contain disclosures similar to PCT/US/01161.

A few of the foregoing methods have been employed to produce Gd-DTPA-containing liposomes for use as contrast agents, and such efforts have been reported in the literature. For example, Unger et al., Radiology, Vol. 157(P), p. 314 (1985) (abstract) (presented at the 71st Radiologic Society of North America Scientific Assembly, Chicago, Illinois, November 19, 1985), examines the use of Gd-DTPA containing liposomes prepared by sonication methods to selectively deliver contrast media to the liver and spleen.

U.S. Patent No. 4,728,575 discloses liposomal vesicles containing paramagnetic materials such as Gd-DTPA which are useful as contrast agents for NMR imaging. According to the patent, the Gd-DTPA-containing vesicles can be prepared using methods such as homogenization, chelate dialysis, sonication, and the like. External Gd-DTPA can be removed from the vesicles by gel filtration, ultrafiltration or similar methods. In the preparatory example shown, the paramagnetic vesicles were formed using sonication methods followed by gel filtration. The disclosures suggest that the liposomal contrast agent

described is targeted to tumor, rather than the normal, tissue, which, if true, would theoretically cause a decrease, not an increase, in contrast between normal and tumor tissue, particularly when employed in the liver.

U.K. Patent Application GB 2193095 A, published February 3, 1988, discusses liposomes containing macromolecule-bound paramagnetic ions such as Gd-DTPA and their use as NMR contrast agents. The liposomes described in the examples were prepared using a sonication-type process.

Despite the theoretical reasons why the liposomally encapsulated contrast agents prepared to date and reported in such publications as Unger et al., Radiology, Vol. 157(P), p. 314 (1985) (abstract) (presented at the 71st Radiologic Society of North America Scientific Assembly, Chicago, Illinois, November 19, 1985), U.S. Patent No. 4,728,575 and U.K. Patent Application GB 2193095 A, should solve the need for acceptable MRI contrast agents for use in detecting hepatic and splenic malignancies, there are in fact substantial problems and/or limitations associated with their use. First, contrast enhancement (differential relaxivity) observed with these products is lower than desirable. In addition, such liposomes have been found to be relatively unstable and prone to leakage of the paramagnetic contrast agents. Moreover, the encapsulation efficiency of the aforementioned liposome/contrast agent systems developed to date is quite low. Furthermore, and quite significantly, toxicity

problems have been encountered. These, as well as other disadvantages, have made the use of such liposomally encapsulated paramagnetic agents less desirable than had been hoped.

It has now been found in the present invention that liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or superparamagnetic agent such as Gd-DTPA provide a significantly better and more useful MRI contrast agent than heretofore known in the art. These contrast agents provide high relaxivity and good contrast enhancement, resulting in a concomitant increase in tumor detection rate. Moreover, these agents show no observable toxicity and are extremely stable, retaining their integrity even after long periods of storage.

It has also been found in the present invention that liposomes, however prepared, having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic and/or superparamagnetic agent provide excellent MRI contrast agents. Surprisingly, these small sized liposomes exhibit superior relativity and contrast enhancement, resulting in a significant increase in tumor detection, and are also particularly effective as blood pool imaging agents.

SUMMARY OF THE INVENTION

The present invention provides contrast agents for magnetic resonance imaging (MRI) which are particularly

useful as blood pool imaging agents and in diagnosing the presence of tumorous tissue in the liver and spleen.

Specifically, the present invention provides MRI contrast agents comprising liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or superparamagnetic agent. In addition, the present invention also provides MRI contrast agents, however prepared, comprising liposomes having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic and/or superparamagnetic agent.

The present invention also includes a method for diagnosing the presence of tumorous tissue in a patient comprising (i) administering to the patient one or more of the foregoing MRI contrast agents, and (ii) scanning the patient using MRI to obtain visible images of any tumorous tissue. The present invention further includes a method of imaging the blood pool of a patient comprising (i) administering to the patient liposomes of Claim 19 and (ii) scanning the patient using MRI to obtain visual images of the location of the blood pool.

The subject invention is described in greater detail below.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention provides MRI contrast agents comprising liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or superparamagnetic agent.

The term "freeze-thaw extrusion" as used herein, denotes the use of a process wherein liposomes are subjected to one or more cycles of freezing and thawing, followed by one or more passages through an extruder. Specifically, the freeze-thaw extrusion process may be carried out as follows.

First, liposomes containing paramagnetic and/or super-paramagnetic agents are initially prepared using any one of a variety of conventional liposome preparatory techniques. As will be readily apparent to those skilled in the art, such conventional techniques include sonication, chelate dialysis, homogenization, solvent infusion coupled with extrusion, as well as others. These techniques, as well as others, are discussed, for example, in U.S. Patent No. 4,728,578, U.K. Patent Application GB 2193095 A, U.S. Patent No. 4,728,575, U.S. Patent No. 4,737,323, International Application PCT/US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), U.S. Patent No. 4,533,254, Mayhew et al., Methods in Enzymology, Vol. 149, pp. 64-77 (1987), Mayhew et al., Biochimica et Biophysica Acta, Vol. 775, pp. 169-174 (1984) and Cheng et al., Investigative Radiology, Vol. 22, pp. 47-55 (1987). The disclosures of each of these publications are incorporated by reference herein, in their entirety. As a preferred technique, a solvent free system similar to that described in International Application PCT/US85/01161, is employed in

initially preparing the liposome constructions. Using this technique, a lipid powder or pellet is placed in an aqueous buffer solution containing the paramagnetic and/or super-paramagnetic agents of the present invention.

The liposomes thus prepared are then subjected to at least one freeze-thaw cycle, that is, freezing followed by thawing. In the freezing portion of the cycle, the liposomes are placed in suitable vials which are then subjected to liquid nitrogen, preferably for about 30 seconds to about 5 minutes. Although liquid nitrogen is the preferred cooling medium, other cooling procedures may also be used. For example, the vials with liposomes may be placed in a freezer at a temperature of preferably about -20°C to about -90°C for preferably up to about one hour. Moreover, cooling mixtures such as acetone/dry ice, methanol/dry ice or ethanol/dry ice and the like may be used as an alternative to liquid nitrogen.

The thawing portion of the cycle is carried out by transferring frozen liposome-containing vials from the cooling medium, to a medium having a temperature greater than about 0°C. Typically, the vials are placed in warm water between about 30°C and about 70°C. The temperature is not critical to the thawing, but it is important that the vials are held at the elevated temperature until the liposome suspension has completely thawed.

This entire cycle of freezing and thawing is performed at least one and up to about ten times, or more.

After completion of the freeze-thaw cycle(s), the thawed liposomes are then passed at least one and up to about twenty times, or more, under pressure through an extruder having a filter with pore size of about 15 to about 600 nm diameter. More than one filter may be used, in, for example, a series alignment. The pressure may be applied in the form of nitrogen, argon, or other inert gas, or, alternatively, may be applied by mechanical pump or direct displacement. As one skilled in the art would recognize, the size of the filter pore determines the relative size, lamellarity, trap volume and size distribution of the resultant lipid vesicles. As will be apparent to those in the art, the smaller the pore size, the smaller the liposome size, trap volume and size distribution, and the less the lamellarity. It should be noted that the application of heat to the extruder device, either from an external source (heat plate, heat gun or the like) or by means of a thermostatted jacket, renders the lipid membranes more deformable and allows extrusion to take place at lower pressures. In addition, by applying such heat, smaller sized vesicles can be more easily produced. It is preferable to extrude lipid vesicles at temperatures at which their lipids are in the liquid-crystal rather than gel state, to facilitate extrusion of the liposomes. Suitable temperatures will be readily apparent to those skilled in the art, by reference to standard lipid texts.

Although filter pore sizes of about 15 to about 600 nm may be employed, preferable filter pore sizes are about 15 to about 30 nm which will generally yield liposomes in the range of about 20 to about 50 nm in diameter. More preferably, pore sizes of about 15 to about 25 nm, generally yielding liposomes of about 20 to about 30 nm in diameter, most preferably pore sizes of about 25 nm, generally yielding liposomes of about 35 nm in diameter, are utilized. The smaller pore sizes are preferred since they yield correspondingly smaller liposomes as well as liposomes with correspondingly less lamellarity. It has been found that the smaller liposomes exhibit greater relaxivity and/or show greater effectiveness at improving contrast. This greater relaxivity is believed to be due to the presence of a proportionally larger surface area relative to the internal volume, which permits a greater diffusion of water across the lipid membrane and thus better contrast enhancement. Significantly, relaxivity has been found to bear a direct linear relationship with 1/r, wherein r is radius. In addition, the smaller liposomes have been found to exhibit longer circulation half-lives. Further, it has been found that the less lamellar liposome vesicles, particularly the unilamellar liposome vesicles, also have been found to exhibit a higher relaxivity, presumably because, with less lamellarity, a higher rate of water transport across the lipid membrane can also occur.

If desired, the liposomes can be extruded, then freeze-thawed, in addition to being extruded subsequent to

the freeze-thaw cycle. The entire freeze-thaw extrusion process can be carried out as many times as desirable. Freeze-thaw extrusion techniques as generally employed in the preparation of liposomes are known in the art, and are described, for example, in International Application PCT/US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), and Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), the disclosures of which are all hereby incorporated herein by reference in their entirety. Such techniques may be utilized in the preparation of the freeze-thawed paramagnetic and superparamagnetic encapsulating liposomes of the present invention.

In a second embodiment, the present invention provides MRI contrast agents comprising liposomes having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic and/or superparamagnetic agent.

The liposomes of the latter embodiment may be prepared using any one of a variety of conventional preparatory techniques, provided that the liposomes so produced have a diameter of less than about 50 nm. As will be readily apparent to those skilled in the art such conventional techniques include sonication, chelate dialysis, homogenization, solvent infusion coupled with extrusion, freeze-thaw extrusion, microemulsification, as well as others. These techniques are discussed, for example, in U.S. Patent No. 4,728,578, U.K. Patent Application GB 2193095 A, U.S. Patent No. 4,728,575, U.S.

Patent No. 4,737,323, International Application PCT/US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), U.S. Patent No. 4,533,254, Mayhew et al, Methods in Enzymology, Vol. 149, pp. 64-77 (1987), Mayhew et al., Biochimica et Biophysica Acta, Vol. 775, pp. 169-74 (1984), and Cheng et al, Investigative Radiology, Vol. 22, pp. 47-55 (1987), all incorporated herein by reference, in their entirety. As preferred techniques for the preparation of liposomes having a diameter of less than 50 nm, microemulsification or freeze-thaw extrusion (particularly a solvent free freeze-thaw extrusion technique) is employed. Most preferably microemulsification is utilized. Microemulsification is particularly preferred because of its suitability for large scale production of liposomal radiologic contrast agents.

Freeze-thaw procedures have been described in detail herein, and include those procedures described, for example, in International Application PCT-US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), and Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), all incorporated herein by reference, in their entirety.

Microemulsification techniques are discussed in U.S. Patent No. 4,533,254, Mayhew et al., Methods in Enzymology, Vol. 149, pp. 64-77 (1987), Mayhew et al., Biochimica et Biophysica Acta, Vol. 775, and Cheng et al.,

Investigative Radiology, Vol. 22, pp. 47-55 (1987), all incorporated herein by reference, in their entirety. Specifically, the microemulsion process may be carried out as follows.

First, liposomes containing paramagnetic and/or superparamagnetic agents are initially prepared using any one of a variety of conventional liposomes preparatory techniques. As will be readily apparent to those skilled in the art, such conventional techniques include sonication, chelate dialysis, homogenization, solvent infusion coupled with extrusion, as well as others. These techniques, as well as others, are discussed, for example, in U.S. Patent No. 4,728,578, U.K. Patent Application GB 2193095 A, U.S. Patent No. 4,728,575, U.S. Patent No. 4,737,323, International Application PCT/US85/01161, Mayer et al., Biochimica et Biophysica Acta, Vol. 858, pp. 161-168 (1986), Hope et al., Biochimica et Biophysica Acta, Vol. 812, pp. 55-65 (1985), U.S. Patent No. 4,533,254, Mayhew et al., Methods in Enzymology, Vol. 149, pp. 64-77 (1987), Mayhew et al., Biochimica et Biophysica Acta, Vol. 775, pp. 169-174 (1984), and Cheng et al., Investigative Radiology, Vol. 22, pp. 47-55 (1987), all incorporated herein by reference, in their entirety.

The liposome composition thus prepared is then subjected to a high pressure impingement emulsifier, such as the Microfluidizer®, an apparatus available from Microfluidics Corporation, a subsidiary of Biotechnology Development Corporation, located in Newton, Massachusetts.

The Microfluidizer emulsifier is described and claimed in U.S. Patent No. 4,855,165, the disclosures of which are incorporated by reference herein, in their entirety. The device consists of a high pressure (up to about 23,000 psi) pump and an interaction chamber in which the emulsification takes place. The pump forces the composition into the chamber where the composition is split into two streams which pass at very high velocity through at least two slits and collide resulting in the production of small sized liposomes. As one skilled in the art would recognize, generally multiple passes provide a smaller average liposome size and a narrower particle size distribution. Similarly, higher pressures, with the greater shear and cavitation forces produced thereby, are believed to assist in the formation of smaller average liposome sizes. As one example, a pressure of about 11,000 psi coupled with a total of thirty passes through the Microfluidizer® will produce liposome compositions having a mean liposome size of less than about 50 nm.

Although liposomes having a size of less than 50 nm may be employed in the second embodiment of the subject invention, preferably the liposomes have a minimum size range of about 20 nm, more preferably between 20 and 35 nm, most preferably about 35 nm. It has been found that generally the smaller the liposomes, the greater the relaxivity and/or contrast enhancement and/or the longer the circulation half-life.

The materials which may be utilized in preparing the liposomes of the present invention include any of the materials or combinations thereof known to those skilled in the art as suitable in liposome construction. Such materials include, but are not limited to, lipids such as cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, lysolipids, fatty acids, sphingomyelin, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids, polymerizable lipids, and combinations thereof. As one skilled in the art would recognize, other nonlipid materials such as polysaccharides and other polymers can be used alone, or in combination with, the lipid materials, in preparing the subject liposomes. These materials can be employed in varying combinations and ratios, in accordance with conventional liposome preparatory protocol. Preferable liposome constructions include constructions containing lipid materials, most preferably those comprising about 60 to about 80 mole percent phosphatidylcholine and about 20 to about 40 mole percent cholesterol. Such constructions are most preferred because of their expected superior stability and relaxivity characteristics.

The liposomes of the present invention encapsulate paramagnetic and superparamagnetic agents. As used herein, the phrase "paramagnetic agent" denotes a compound comprising a transition, lanthanide and actinide

element covalently or nonconvalently bound to complexing agents or to proteinaceous macromolecules. The phrase "paramagnetic agent" also denotes stable free radicals. Preferably, the elements are transition and lanthanide elements. Preferable transition and lanthanide elements include Gd(III), Mn(II), Cu(II), Cr(III), Fe(II), Fe(III), Co(II), Er(II), Ni(II), Eu(III) and Dy(III). Suitable complexing agents will be readily apparent to those skilled in the art and include, but are not limited to, diethylene-triamine- pentaacetic acid (DTPA), ethylenediaminetetra-acetic acid (EDTA), 1,4,7,10-tetraazacyclododecane-N,N',N',N'''-tetraacetic acid (DOTA), 1,4,7,10-tetra-azacyclododecane-N,N',N''-triacetic acid (DO3A), 3,6,9-triaza-12-oxa-3,6,9-tricarboxymethylene-10-carboxy-13-phenyl-tridecanoic acid (B-19036), hydroxybenzylethylene-diamine diacetic acid (HBED), N,N'-bis(pyridoxyl-5-phosphate)ethylene diamine, N,N'-diacetate (DPDP), 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA), 1,4,8,11-tetraazacyclotetradecane-N,N'N'',N'''-tetraacetic acid (TETA). Preferable complexing agents are DTPA and DOTA, most preferably DTPA. Suitable proteinaceous macro-molecules include albumin, polyarginine, polylysine, gamma-globulin and beta-globulin. Preferable proteinaceous macromolecules are albumin, polyarginine, polylysine, and polyhistidine. Suitable stable free radicals include stable nitroxides. As used herein, the phrase "superpara-magnetic agent" denotes ferro- or ferrimagnetic compounds, such as magnetite. If desired, vasoactive amines may be

encapsulated along with the paramagnetic and/or superparamagnetic agents.

Preferably, the liposomes of the present invention encapsulate paramagnetic agents. More preferably, the paramagnetic agents are lanthanide elements bound to complexing agents. Even more preferably, the paramagnetic agents are Gd(III)-DTPA or Gd(III)-DOTA. Most preferably, the paramagnetic agents are Gd(III)-DTPA.

The present invention is useful in diagnosing the presence of tumorous tissue in a patient. The patient can be any type of mammal, but most preferably is a human. The process is particularly useful in diagnosing the presence of tumors of the liver and spleen, although tumors in other organs or other regions such as muscle regions may be diagnosed. Bone marrow is also believed to particularly well suited to the use of this contrast agent.

The liposomes of the present invention are also useful in blood pool imaging, that is, imaging of the blood present in the mammalian system. Thus, the subject liposomes can be employed in assessing the vascularity of both healthy and tumors tissues, and accordingly, are useful in evaluating, for example, the pathological conditions of decreased vascular perfusion such as myocardial or cerebral ischemia. As blood pool imaging agents, the liposomes of the present invention are also useful in the determination of blood volume, and the assessment of vascular leakage (including the assessment of vascular pore size), determinations which may be of

importance in the diagnosis and treatment of a variety of diseases including cancer and ischemia.

The diagnostic process of the present invention may be carried out as follows. First, the patient is administered liposomes of the invention, that is, liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or superparamagnetic agent, or liposomes, however prepared, having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic or superparamagnetic agent. The patient (either the entire patient or a particular organ or region of that patient), is then scanned using MRI to obtain visible images of any tumorous tissue or the blood pool of a patient.

As one skilled the art would recognize, administration may be carried out in various fashions, such as intravascularly, intralymphatically, parenterally, subcutaneously, intra-muscularly, or intraperitoneally, using a variety of dosage forms. Preferable routes of administration are intravascular and intralymphatical. The useful dosage to be administered and the mode of administration will vary depending upon the age, weight and mammal to be diagnosed, and the particular liposome construction and paramagnetic and/or superparamagnetic agent employed. Typically, dosage is initiated at lower levels and increased until the desired contrast enhancement is achieved. In carrying out the diagnostic method of the present invention, the liposomes can be used alone in

combination with one another, or in combination with other diagnostic and/or therapeutic agents.

The MR imaging techniques employed are conventional and are described, for example, in D.M. Kean and M.A. Smith, Magnetic Resonance Imaging: Principles and Applications (William and Wilkins, Baltimore 1986). Contemplated MRI techniques include, but are not limited to, nuclear magnetic resonance (NMR) and electron spin resonance (ESR). The preferred imaging modality is NMR.

The present invention is further described in the following Examples. These Examples are not to be construed as limiting the scope of the appended Claims.

Examples

Example 1

Preparation Of Gd-DTPA-Containing Liposomes By Freeze-Thaw Extrusion Techniques and Characterization of the Same.

Unilamellar vesicles were prepared using the freeze-thaw extrusion process as follows. Egg phosphatidylcholine (0.216 g), cholesterol (0.077 g), and wheat germ digalactosyldiglyceride (0.023 g) (molar ratio 55:40:5) were dissolved in 1 ml chloroform stock solution. Fifty μl of a ^{3}H-DPPC (^{3}H-dipalmitoyl phosphatidylcholine) stock solution (0.25 mCi/2.5 ml toluene, NEN Canada) was added, the mixture evaporated initially under nitrogen, then held at reduced pressure for two hours to remove residual solvent.

The lipid mixture was dispersed in 4 ml 0.67 M ^{153}Gd-DTPA pH 7.0, to which had been added 50 μl of ^{14}C-inulin (0.25 mCi/2.5 ml water, NEN Canada), by vortexing at room temperature to yield multilamellar vesicles (MLVs). The MLVs were transferred to a 4.5 ml cryovial, then subjected to five cycles of freeze-thawing using liquid nitrogen. The frozen and thawed MLVs were then sized by ten passes under nitrogen pressure through two stacked 0.4 micron polycarbonate filters (Nucleopore) using an Extruder Device (Lipex Biomembranes). Two thirds of this sized preparation was then passed ten times through two stacked 0.2 micron filters. Half of this latter preparation was then passed ten times through two stacked 0.1 micron filters.

For each of the sized vesicle preparations, external Gd-DTPA was removed by chromatography on Sephadex G50 F (Pharmacia) which had been re-swollen by stirring for two hours at room temperature in saline buffer consisting of 10 mM HEPES, 6 mM KCl, 139 mM NaCl, pH 7.4. G50 F was packed into 23 x 1.5 cm Biorad columns and equilibrated with at least 50 ml of buffer prior to addition of sample. The sample load was 1 ml. Vesicles were eluted with saline buffer in the column void volume and a sample taken for scintillation counting to determine trap volumes as described in Hope et al., Biochimica et Biophysica Acta, Vol. 182, pp. 55-65 (1985). For one batch of 0.4 micron vesicles, non-encapsulated Gd-DTPA was removed by dialysis of the extruded vesicles against 4 liters of saline buffer

at 4°C with constant stirring. The external dialysis medium was changed a total of eight times over 48 hours. ^{14}C-inulin tracer was used to determine encapsulation efficiency.

The size of the 0.4 micron vesicles was assessed by electron microscopy using negative staining techniques.

The lipid and Gd-DTPA concentrations in the different vesicle preparations are shown in Table I. The 0.4 micron vesicles contained the highest concentration of Gd-DTPA. The 0.4 micron vesicle preparations had 39% efficiency for encapsulation of Gd-DTPA and removal of free Gd-DTPA by dialysis resulted in the highest concentration of Gd-DTPA. Electron microscopy of the 0.4 micron vesicles showed a mean diameter of 239 ± 39 nm.

Table I

Characterization of Freeze-Thaw Gd-DTPA Liposomes

Encapsulated Filter Pore Size (nm)	Lipid Conc. moles/ml	Gd-DTPA Conc. moles/ml	Efficiency (%)
100	35	38	16
200	39	42	20
400	44	47	39
400*	116	124	39

*Unbound Gd-DTPA removed by chromatography, dialysis used in all other cases.

Example 2

Analysis Of In Vitro Stability Of Gd-DTPA-Containing Liposomes Prepared By Freeze-Thaw Extrusion Techniques.

In vitro stability of the 0.4 micron liposomes prepared in Example 1 were tested by dialysis performed over 72 hours against normal saline (6 x 1 L) at 4°C. The stability was calculated by counting the ^{153}Gd-DTPA activity remaining in the liposomes at the end of 72 hours.

The vesicles were found to be 100% stable to the prolonged dialysis.

Example 3

Analysis Of In Vivo Contrast Enhancement And Toxicity Of Gd-DTPA-Containing Liposomes Prepared By Freeze-Thaw Extrusion Techniques.

All in vivo imaging was performed on a Siemens 1.5 Tesla magnet (Iselin, N.J.). Spin echo technique was utilized for both T1- and T2-weighted imaging. T1-weighted sequences utilized parameters TR/TE: 400 msec/16 msec, 4 acquisitions, 256 x 256 matrix 16 cm field view for 2 mm thick slices. For T1 calculations, images were acquired using a fixed TE of 28 msec with TR varying from 300, 500, 1500 and 3500 msec. For T2 calculations a fixed TR of 3500 msec was used with TE values of 28, 45, 70, 90 and 150 msec. T1 and T2 calculations were preformed using the software on the Magnetom System (Iselin, N.J.) by selecting a region of interest on the CRT monitor.

Male buffalo rats, weighing approximately 450 grams, were scanned using a 7.5 cm circular surface coil. The animals were scanned in the supine position, and signal intensities of liver, paraspinal muscle, kidney, renal pelvis, bladder and vascular structures were measured

and post contrast. Rats were imaged pre and post contrast injection of either 0.4 micron Gd-DTPA liposomes (8 rats) or free Gd-DTPA (2 rats).

Injections of the 0.4 micron liposomes prepared in Example 1 were performed by tail vein injection using a 26 gauge butterfly needle. Gd-DTPA liposomes were injected over a period of approximately two minutes; liposomes were diluted with an equal volume of normal saline immediately prior to injection. After imaging, animals were observed for eight weeks for any signs of delayed toxicity.

Scans of rats pre and post contrast with Gd-DTPA liposomes showed enhancement of liver, renal cortex and vascular structures. A dose of 0.1 millimoles/kg or greater of Gd-DTPA in liposomes resulted in a dramatic increase in signal intensity (S.I.) as shown in Table II. Maximal enhancement was observed immediately after injection of contrast agent, but enhancement of liver and vascular structures was sustained for two hours after contrast in two animals imaged two hours after injection of liposomal Gd-DTPA. Scans of two control animals after injection of 0.1 and 0.2 millimoles/kg of free Gd-DTPA showed enhancement of renal cortex, renal pelvis and urinary bladder, but no appreciable enhancement of liver or vascular structures.

During I.V. injection of Gd-DTPA liposomes there was no sign of respiratory distress or other toxicity in the rats. The rats were observed for two months after injection of liposomes and then sacrificed and showed no

evidence of toxicity from Gd-DTPA liposomes either at time of injection or on delayed follow-up and gross pathologic examination.

Table II

Dose Gd-DTPA Freeze-Thaw Liposomes and Contrast Enhancement

Dose (mM/Kg)	Liver	Muscle	Liver + Muscle
0.054 lipo (n=1)	5.2%	3.9%	1.3%
0.080 lipo (n=1)	71.1%	31.0%	30.6%
0.100 lipo* (n=5)	10.0 to 71.1% x = 25.0%	x = -11.6%	x = 39.0%
0.130 lipo (n=1)	29.0%	-46.2%	239.8%
0.254 lipo (n=1)	232.0%	151.4%	153.2%
0.109 Free Gd-DTPA	0.0%	22.4%	-19.3%
0.218 Free Gd-DTPA	11.6%	3.1%	8.2%

Liver enhancement: (liver S.I. post - liver S.I. pre) + (liver S.I. pre). Muscle enhancement (paraspinal muscle): (muscle S.I. post - muscle S.I. pre) + muscle pre. Liver + muscle refers to ratio of liver enhancement to muscle enhancement. Post-contrast S.I. measured from scans 30 min after I.V. injection of contrast agents. S.I. = signal intensity, lipo = liposomal Gd-DTPA, x = mean. *Note that 5 rats were injected with dose of 0.1 millimoles Gd-DTPA/kg encapsulated within liposomes and mean values for enhancement are depicted.

Example 4

Preparation And In Vitro Characterization Of Gd-DTPA-Containing Liposomes Prepared By Freeze-Thaw Extrusion Techniques.

Preparation:

Unilamellar vesicles were prepared by extrusion through polycarbonate filters under moderate pressure using the procedures set forth in Hope et al., Biochimica et Biophysica Acta, Vol. 182, pp. 55-65 (1985). Vesicles were composed of egg phosphatidylcholine (PC), either alone, or in combination with 40 mole percent cholesterol. PC was obtained from Avanti Polar Lipids and cholesterol from Sigma; both were used without further purification. Typically, 3.6 mmoles (2.83 g) of PC and 2.4 mmoles (0.93 g) of cholesterol were dissolved together in a minimum volume of chloroform in a 250 ml round-bottom flask to which was added 50 μl of ^{3}H-dipalmitoyl-phosphatidylcholine (DPPC) stock solution (250 μCi/2.5 ml toluene, NEN, Canada). The purpose of the ^{3}H-DPPC is to serve as a means of quantitation of the lipid concentration. The chloroform was removed initially by rotary evaporation under reduced pressure to leave a thin film on the walls of the flasks, then the contents held under reduced pressure (< 0.1 mm Hg) for at least two hours to remove residual solvent. The lipid was dispersed by vigorous shaking at room temperature in 20 ml of 0.67 M Gd-DTPA, pH 7 (sodium salt), labeled with 5 μl of ^{14}C-inulin (250 μCi/2.5 ml water, NEN, Canada). The ^{14}C-inulin served as a aqueous marker and permitted a

facile determination of the trap volumes of the vesicles. The multilamellar vesicles formed upon dispersion were transferred to cryovials then quench-frozen in liquid nitrogen. The cryovials were then placed in warm water until the lipid suspension had completely thawed. This cycle of freezing and thawing was repeated a further four times. The frozen and thawed vesicles were then sized by ten passes under nitrogen pressure (< 100 psi) through two stacked 0.4 micron polycarbonate filters (Nucleopore) using the Extruder Device (Lipex Biomembranes, Canada) to produce liposomes having a mean of about 400 nm. A portion of this sized preparation was then passed ten times through two stacked 0.2 micron filters to produce liposomes having a mean of about 200 nm. A portion of this latter preparation was then passed a further ten times through either two stacked 0.1 or 0.05 micron filters to produce liposomes having means about 100 and 70 nm, respectively. An aliquot of each of the sized preparations was removed for scintillation counting to determine the ^{3}H-DPPC and ^{14}C-inulin content.

For each of the sized vesicles, external untrapped Gd-DTPA was removed by exhaustive dialysis at 4°C against 4 l of saline buffer (10 m moles 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 6 mM KCl, 139 mM NaCl, pH 7.4). The external dialysis medium was changed a total of eight times (8 x 4 l) over 2-3 days. The size of the dialyzed particles were determined by quasielastic light scattering using a Nicomp Model 270

particle sizer operating at 634.2 nm by standard cumulants analysis. The entrapment efficiencies of the vesicles were determined by chromatography of an aliquot of the dialyzed vesicles on Sephadex G50 F (Pharmacia), equilibrated with saline buffer. Vesicles with entrapped ^{14}C-inulin was retained on the column. A sample of the eluted vesicles was taken for dual-label scintillation counting as before. Vesicles were stored at 4°C until further use.

Phantoms were prepared by diluting the vesicle preparation with Dulbecco's phosphate buffered saline (PBS) (Gibco), so as to obtain several samples for each vesicle preparation with an effective concentration of Gd-DTPA in the range 0.2-2 mmoles. Approximately 10 ml of each dilution was placed in 20 ml plastic syringes (Becton Dickinson), which were sealed with parafilm to prevent leakage and placed in a custom-built acrylic phantom holder which positioned the syringes with their long-axes horizontal within the bore of the magnet.

Imaging:

Phantoms were imaged using a Siemens 1.5 Tesla Magnetom whole body scanner (Iselin, N.J.) using body coil. For T1 calculations, images were acquired using spin-echo sequences with a fixed TE of 17 msec and TR values of 100, 300, 450, 600, 900, 1200, 1800, 2500 and 3500 msec. Other parameters were 4 acquisitions, 256 x 256 matrix, 30 cm field of view and 10 mm slices.

Vesicle Stability (Retention):

In vitro stability (retention) of the vesicle preparations in saline was determined at various times after preparation by dialysis (Spectrapor 10 mm tubing, M.W. cutoff 12-14,000) over 120 hours against 5 x 11 changes of normal saline at 4°C. The stability was calculated on the basis of either the percentage of ^{153}Gd-DTPA activity remaining inside appropriately labelled vesicles or by determination of the ^{3}H-DPPC/^{14}C-inulin ratio by scintillation counting. The results are shown in Table III.

Table III

Characteristics of Gd-DTPA-Containing Freeze-Thaw PC:Cholesterol (6:4) Liposomes

Diameter (nm)[1] Mean ± S.D.	Trap Volume[2] (μl/mole)	Lipid Conc.[3] (μmole/ml)	[Gd]eff[4] (μmole/ml)	Trapping[5] Efficiency (%)	Lipid Dose[6] mg/kg	Percentage[7] Retention	Relaxivity[8] (sec^{-1} x mM^{-1})
Free Gd-DTPA	N/A	N/A	N/A	N/A	N/A	N/A	2.79
400 ± 40	1.95	181	236	35	95	100	0.42
200 ± 30	1.17	175	137	20	140	94	0.51
100 ± 20	0.87	185	108	16	214	100	1.05
70 ± 20	0.71	169	79	12	267	100	1.60

1. Based on quasielastic light scattering.
2. Trap Volume = T = μl Gd-DTPA per μmole of lipid.
3. Lipid concentrations (L) are typical values for preparations used in this study.
4. Effective concentration of Gd-DTPA = T x L x [Gd]int) - 1000. In this study [Gd]int was 670 μmoles/ml.
5. Trapping efficiency = [Gd]eff x 100/[Gd]int.
6. Lipid dose = dose of lipid required to give a [Gd]eff of 0.2 millimole/kg. For PC:Cholesterol (6:4) mixtures, the average M.W. was taken as 626.
7. Percentage retention of internal contents after 4 months storage in saline buffer at 4°C.
8. In vitro relaxitivity = sec^{-1} x mM^{-1}.

Example 5

Preparation and *In Vitro* Characterization of Gd-DTPA-Containing Liposomes Having Different Ratios of Egg Phosphatidylcholine and Cholesterol.

Vesicles were prepared substantially as described in Example 4, except that varying amounts of egg phosphatidylcholine and cholesterol were employed.

Imaging was also carried out substantially as described in Example 4, except that in addition to the imaging done as a Siemans 1.5 Tesla Magnetom scanner, imaging was also carried out on a Toshiba MR-50A 0.5 Telsa whole body scanner.

The results are shown in Table IV. The results reveal that the greater the proportion of cholesterol as compared to phosphatidylcholine, the lower the relaxivity and the lower the effectiveness of the liposomes as contrast agents.

Table IV

Relaxivity of Gd-DTPA-Containing Freeze-Thaw PC:Cholesterol Liposomes

Mole% Cholesterol	Average Vesicle Diameter (mM^{-1}) 170	100	70
0.5T	Relaxivity ($sec^{-1} \times m\mu^{-1}$)*		
0	1.399 (.178)	1.730 (.189)	2.850 (.426)
10	1.294 (.174)	1.791 (.216)	2.618 (.334)
20	1.405 (.218)	1.878).219	2.556 (.286)
30	0.885 (.162)	1.557 (.229)	2.241 (.261)
40	0.807 (.169)	1.298 (.188)	1.864 (.214)
50	0.549 (.184)	0.880 (.220)	1.354 (.215)
1.5%			
10	1.105 (.060)	1.697 (.097)	2.034 (.088)
20	1.039 (.074)	1.627 (.105)	1.992 (.117)
30	0.721 (.054)	1.209 (.063)	1.830 (.087)
40	0.712 (.065)	1.017 (.077)	1.526 (.097)
50	0.361 (.059)	0.794 (.043)	1.000 (.049)

*Relaxivity values are expressed as the mean (± standard deviation) based on a linear regression to four values of the effective Gd-DPTA concentration.

Example 6

Preparation and Biodistribution Characterization of Gd-DTPA-Containing Liposomes Prepared By Freeze-Thaw Extrusion Techniques.

Preparation:

Unilamellar vesicles were prepared substantially as described in Example 4, except that about 100μci of ^{153}Gd-DTPA was employed as part of the Gd-DTPA used, and no DPPL was used.

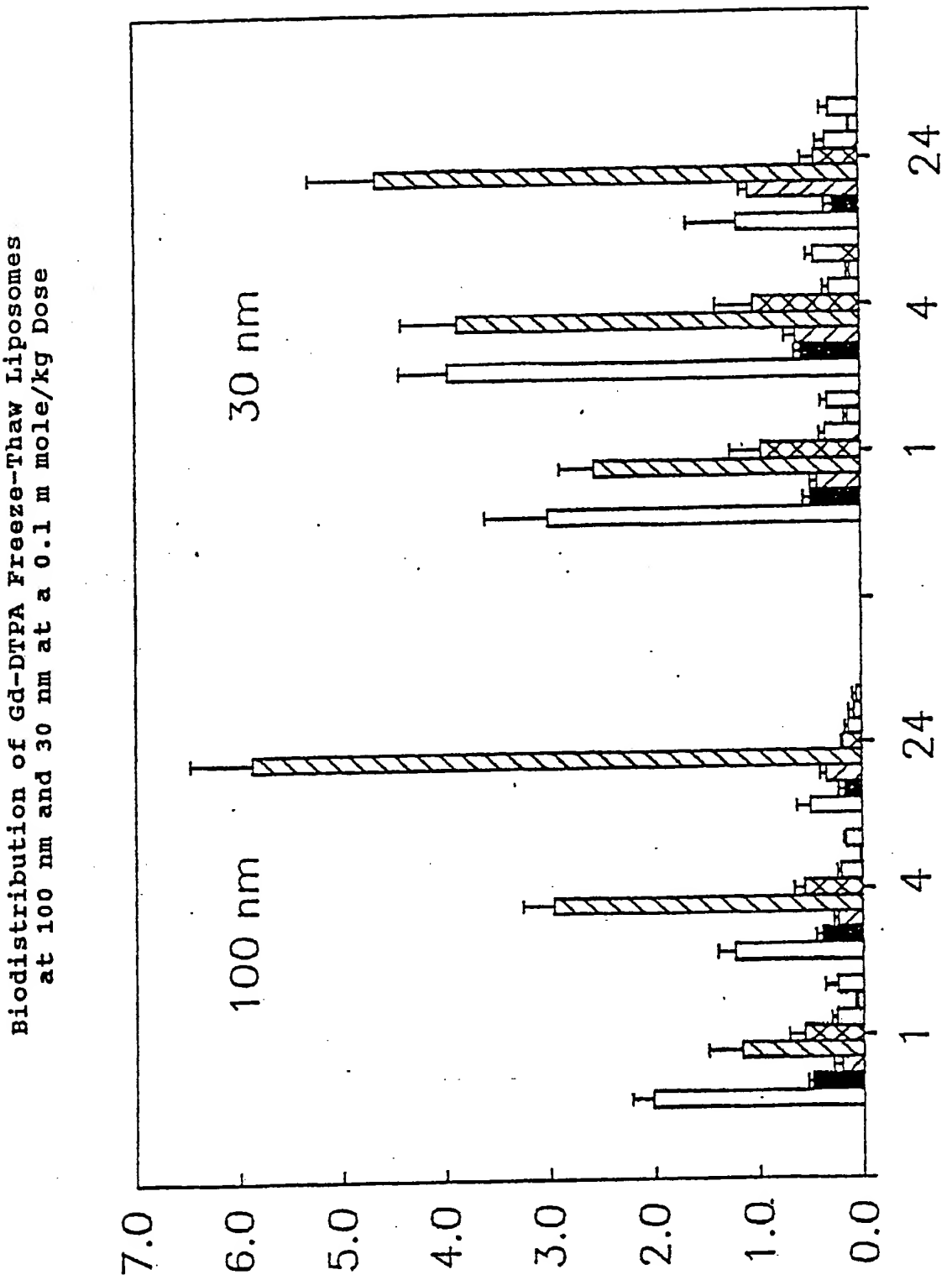
Biodistribution:

Male Fischer 344 rats weighing approximately 250 g were used in the biodistribution studies. Rats were anesthetized in all experiments with intramuscular injection of Rompun® (Haver, Kansas City), 0.07 ml/250 g body weight plus Ketamine® (Parke-Davis, Morris Plains, N.J.), 0.05 ml/250 g body weight. All contrast injections were performed via tail vein over approximately 1 minute.

The biodistribution of both 100 nm and 30 nm diameter lipid vesicles with entrapped Gd-DTPA was examined in the 250 g male Fischer rats. Three to five animals were injected intravenously with a total dose of 0.1 millimole/kg of Gd-DTPA. Rats were sacrificed at 1 hour, 4 hours and 24 hours post-administration. Blood, heart, liver, spleen, muscle, lung, kidney, muscle and fat samples were removed and placed in tared empty gamma counting tubes, reweighed and counted. Results were expressed in terms of percentage total injected dose per gram wet-weight tissue.

The results are reported in Figure 1. The results shown biodistribution of ^{153}Gd-DTPA liposomes post-administration for vesicles of 100 nm and 30 nm average diameter given at a dose of 0.1 m mole/kg of Gd-DTPA and ^{153}Gd-DTPA. The bars in the Figure are grouped in sets of eight at each of the 1, 4 and 24 hour intervals. From left to right at each interval is reported the biodistribution data for the blood, heart, liver, spleen, lung, kidney, muscle, and fat. As the results reveal, the smaller liposomes showed a higher uptake by the liver, spleen, lung, muscle, and bone marrow, and a significantly longer blood pool phase. Thus, the smaller vesicles are superior in terms of biodistribution characteristics.

FIGURE 1


Biodistribution of Gd-DTPA Freeze-Thaw Liposomes
at 100 nm and 30 nm at a 0.1 m mole/kg Dose
Biodistribution
(% Total Counts
Per Gram)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
100 nm
30 nm
1
4
24
1
4
24
Time (Hrs.)

Example 7

In Vivo Contrast Enhancement Of Hepatic Metastases By Gd-DTPA-Containing Liposomes Prepared By Freeze-Thaw Extrusion Techniques.

Cell Lines and Tumor Model:

The cell line employed, C5, is a clonal derivative of a rat liver epithelial cell line initially derived from the livers of newborn Fisher rats and subsequently transformed in vitro by transfection with a plasmid containing the T24ras gene driven by the mouse metallothionein promotor. See, Li et al., P.N.A.S., Vol. 35, pp. 344 (1988); Reynolds et al., Oncogene, Vol. 323, pp. 323-330 (1987). It has been shown to grow rapidly in vivo in subcutaneous tissue, intraperitoneally and intrahepatically.

The tumors used in this study were generated by injecting 3 x 10^5 cells into the superficial lobe of rat livers. Three separate injections of 10^5 cells were made within an approximate area of 0.5 cm^2 under direct visualization. The tumors were allowed to grow for 14 to 17 days before imaging followed by sacrifice.

Liposome Preparation:

Egg phosphatidyl choline (EPC) cholesterol (Chol) vesicles (molar ratio 6:4) were prepared by extrusion as described in Example 4.

Imaging:

MR imaging was performed on a whole body 1.5 T Siemens Magnetom (Erlangen, West Germany) using a 15 cm

Helmholtz receive only coil. Spin echo technique was used with TR/TE of 400 msec/17 msec, 2 mm slices with 4 acquisitions. Images were obtained pre and post contrast in both axial and coronal planes with post contrast images obtained from 5 to 60 minutes post contrast.

Rats were lightly sedated with ether and then fully sedated with I.P. Ketamine/Rompun 0.10 ml/100 g body weight. Contrast injections were performed by tail vein over two minutes with volume approximately 0.5 ml. Doses were as follows: free Gd-DTPA 0.5 and 0.2 mM/kg, 400 nm Gd-DTPA liposomes 0.5, 0.2 and 0.1 mM/Kg and 70 nm Gd-DTPA liposomes 0.5 and 0.2 mM/Kg with one rat injected with each dose.

Image Analysis:

Pre and post contrast images were separated and, in a blind study, five radiologists experienced in interpreting MR and oncologic imaging were given the MR images to interpret. The MR scans were presented to the radiologists randomly without knowledge of path findings or contrast agent used. The radiologists were instructed to count the number of metastases, plot lesion locations and measure lesion size. The number and sizes of lesions detected were then correlated with the histologic sections and the true number of metastases detected was determined for pre and post contrast scans. The Mann-Whitney test was used to test for statistical significance of results for lesion detection pre and post contrast.

Histology:

Immediately after imaging, the rats were sacrificed by ether overdose and the livers removed and fixed in 10% formalin. The gross liver specimens were photographed and sectioned in the coronal plant at 2 mm intervals. The number of lesions were counted and size of lesions were measured to correlate with findings on MR images. Microscopic sections were prepared using hematoxylin and eosin stain and studied with light microscopy.

Detection of Hepatic Metastases:

The accuracy of detection of lesions pre and post contrast after Gd-DTPA is shown in Table V. Table V shows that liposomal Gd-DTPA caused a statistically significant improvement in lesion detection by the five radiologists. Free Gd-DTPA caused a statistically significant reduction in lesion detection.

Table V

Blind Study Detection of Hepatic Metastases
Pre and Post Contrast by 5 Radiologists

e	#Path Proven	#Pre	#Post	Sensitivity Pre	Sensitivity Post	Accuracy Pre	Accuracy Post
mm/kg nm	4	1.83±.41	4.00±0***	45.8±10.2%	100±0%	37.5±13.7%	100±0%
mm/kg nm	6	4.80+.84	6.00±0*	80.0±13.9%	100±0%	80.0±13.9%	100±0%
mm/kg nm	7	3.40±.55	6.17±.98***	48.6±7.8%	85.7±14.3%	45.7±11.9%	85.7±14.3%
mm/kg nm	4	2.00±1.41	2.80±.84	50.0±35.3%	70±20.9%	50.0±35.3%	70.0±20.9%
mm/kg nm	2	1.17±.41	2.00±0*	58.3±20.4%	100±0%	58.3±20.4%	100±0%
mm/kg e	7	5.00±.63	2.80±1.64**	71.4±10.1%	40.0±23.5%	71.4±10.1%	40.0±23.5%
mm/kg e	2	1.60±.55	0±0*** isointense	80.0±27.4%	0±0%	70.0±27.4%	0±0%

ends: #Path Proven = number of metastases proven on histology, #Pre and #Post refer to num- of metastases detected on Pre and Post contrast scans. Sensitivity Pre and Sensitivity Post ercentage of lesions detected on Pre and Post contrast scans respectively. Accuracy Pre and t include adjust-ment for false positives. Statistical significance: * = $p < .05$, ** = $p <$, *** = $p < .005$. Same p values correspond for differences between # of metastases detected and Post contrast as well as sensitivity and accuracy. Note that free Gd-DTPA(free) caused nificant reduction in detection of metastases.

Example 8

Preparation Of Gd-DTPA-Containing Liposomes By Micro-emulsification Techniques And Size Characterization Of The Same.

Unilamellar vesicles were prepared using the microemulsification process as follows. Egg phosphatidyl-choline (20 g) and cholesterol (2.46 g) were combined in chloroform. The solvent was removed initially under rotary evaporation to form a thin film on the wall of a round-bottom flask. The flask and contents were then left under reduced pressure (< 0.1 mm Hg) for about 18 hours. The dry lipids were rehydrated in Gd-DTPA (670 mM) by shaking at room temperature to form a dispersion having a concentration of approximately 300 μmoles/ml as measured by lipid phosphorous.

The Microfluidics® M-100 T emulsifier was cleaned by passing approximately 100 ml of ethanol through the device, followed by 200 ml of water, followed by 200 ml of dialysis buffer (10 mM HEPES, 139 mM NaCl, 6 mM KCl, pH 7.4). The pump pressure was set at about 11,000 psi, yielding a flow rate of approximately 200 ml/min, and the lipid dispersion passed through the Microfluidizer® with water cooling. The product was collected and then recycled through the device for a total of 30 passes.

The size distribution of the vesicles was determined by quasielastic light-scattering using a Nicomp Model 270 particle sizer operating at 634.2 nm by standard cumulants analysis.

The results are shown in Table VI. As the results indicate, the majority of the liposomes prepared have a diameter of between about 42.8 and 52.1 nm.

Table VI

Size Characterization of
Microemulsified Gd-DTPA Liposomes

d/nm[1]	Relative Area[2]
21.0[3]	78.6[3]
21.4	82.7
21.8	84.7
42.8	29.0
44.4	93.3
46.1	99.9
48.0	76.4
50.0	18.3
52.1	8.8
171.4[4]	9.0[4]
200.0[4]	16.6[4]
240.0[4]	20.8[4]
300.0[4]	12.8[4]
400.0[4]	5.2[4]
600.0[4]	0.4[4]

[1]d/nm = liposome diameter in nanometers.

[2]Relative Area = total area, relative to the maximum of 46.1 nm, occupied by liposomes having the specified diameters.

[3]Data is believed to represent a machine artifact and not liposomes.

[4]Data is believed to represent either aggregates of liposomes and/or dust particles.

Example 9

Preparation of Gd-DTPA-Containing Liposomes By Microemulsification Techniques And Analysis Of In Vivo Contrast Enhancement.

Preparation:

Unilamellar vesicles were prepared using the microemulsification process as follows. Egg phosphatidylcholine and cholesterol were combined in a ratio of 8:2 in chloroform. The solvent was removed initially under rotary evaporation to form a thin film on the wall of a round-bottom flask. The flask and contents were then left under reduced pressure (< 0.1 mm Hg) for about 18 hours. The dry lipids were rehydrated in Gd-DTPA (670 mM) by shaking at room temperature to form a dispersion that having a concentration of approximately 300 μmoles/ml as measured by lipid phosphorous.

The Microfluidics® M-100 T emulsifier was cleaned by passing approximately 100 ml of ethanol through the device, followed by 200 ml of water, followed by 200 ml of dialysis buffer (10 mM HEPES, 139 mM NaCl, 6 mM KCl, pH 7.4). The pump pressure was set at about 11,000 psi, yielding a flow rate of approximately 200 ml/min, and the lipid dispersion passed through the Microfluidizer® with water cooling. The product was collected and then recycled through the device for a total of 30 passes.

The size distribution of the vesicles was determined by quasielastic light-scattering using a Nicomp Model 270 particle sizer operating at 634.2 nm by standard

cumulants analysis. Liposomes having a mean diameter of less than about 50 nm were obtained.

Imaging:

All in vivo imaging was performed on a Toshiba MR-50A 0.5 Tesla whole body scanner. Scan parameters were TR = 400 msec TE = 15 msec, 5 mm slice, 1 mm interslice gap, 15 cm field of view with 4 acquisitions using a receive-only surface coil.

Male Wistar rats were scanned in the supine position, and signal intensities of liver, kidney and paraspinal muscle as well as urine, were measured pre and post contrast, with post contrast intervals comprising 15 minutes, 1 hour, 4 hours, 24 hours and 48 hours.

Injections of liposomes prepared as described above were made at concentrations of 0.1, 0.05 and 0.025 mM/kg, based on the weight of the rats, and were carried out by tail vein injection using a 26 gauge butterfly needle. The liposomes were injected over a period of approximately two minutes, and were first diluted with an equal volume of normal saline immediately prior to injection.

Scans of rats pre and post contrast showed enhancement of the liver, kidney and urine. The results, reported as a tissue to muscle ratio, are shown in Figures 2-4. As a control, scans of rats injected with free Gd-DTPA at a dose of 0.1 mmoles/kg are reported in Figure 5. The results for the control show no significant enhancement of the liver, kidney and urine.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended Claims.

CLAIMS

What is claimed is:

1. An MRI contrast agent comprising liposomes prepared by a freeze-thaw extrusion process and having encapsulated therein a paramagnetic and/or superparamagnetic agent, said liposomes having a diameter of less than about 50 nm.

2. An MRI contrast agent of Claim 1 wherein said liposomes are prepared by a solvent free freeze-thaw extrusion process.

3. An MRI contrast agent of Claim 1 wherein said liposomes are comprised of lipid materials selected from the group consisting of cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, lysolipids, fatty acids, sphingomyelin, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids, polymerizable lipids, and combinations thereof.

4. An MRI contrast agent of Claim 3 wherein said liposomes are comprised of phosphatidyl choline and cholesterol.

5. An MRI contrast agent of Claim 4 wherein said liposomes comprise about 60 to about 80 mole percent phosphatidyl choline and about 20 to about 40 mole percent cholesterol.

6. An MRI contrast agent of Claim 1 wherein said liposomes are selected from the size range consisting of about 20 to less than about 50 nm.

7. An MRI contrast agent of Claim 6 wherein said liposome size range is about 20 to about 35 nm.

8. An MRI contrast agent of Claim 7 wherein said liposome size is about 35 nm.

9. An MRI contrast agent of Claim 1 wherein said liposomes encapsulate therein a paramagnetic agent.

10. An MRI contrast agent of Claim 9 wherein said paramagnetic agent is a lanthanide or transition element bound to a complexing agent.

11. An MRI contrast agent of Claim 10 wherein said lanthanide or transition elements are selected from the group consisting of Gd(III), Mn(II), Cu(II), Cr(III), Fe(II), Fe(III), Co(II), Er(II), Ni(II), Eu(III) and Dy(III).

12. An MRI contrast agent of Claim 10 wherein said complexing agent is selected from the group consisting of DTPA, EDTA, DOTA, DO3A, B-19036, HBED, DPDP, NOTA and TETA.

13. An MRI contrast agent of Claim 10 wherein said lanthanide element is Gd(III) and said complexing agent is DTPA.

14. An MRI contrast agent of Claim 1 which is a NMR contrast agent.

15. An MRI contrast agent of Claim 1 which is an MRI contrast agent for use in a liver or spleen.

16. A method for diagnosing the presence of tumorous tissue in a patient comprising (i) administering to the patient liposomes of Claim 1 and (ii) scanning the patient using MRI to obtain visible images of any tumorous tissue.

17. A method of Claim 16 wherein the MRI used is NMR.

18. A method of Claim 16 wherein the method is used to diagnose the presence of tumorous tissue in the liver and spleen of said patient.

19. An MRI contrast agent comprising liposomes having a diameter of less than about 50 nm and having encapsulated therein a paramagnetic and/or superparamagnetic agent.

20. An MRI contrast agent of Claim 19 wherein said liposomes are prepared by a microemulsification process.

21. An MRI contrast agent of Claim 19 wherein said liposomes are prepared by a freeze-thaw extrusion process.

22. An MRI contrast agent of Claim 19 wherein said liposomes are comprised of lipid materials selected from the group consisting of cholesterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, phosphatidylinositol, lysolipids, fatty acids, sphingomyelin, glycosphingolipids, glucolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids, polymerizable lipids, and combinations thereof.

23. An MRI contrast agent of Claim 22 wherein said liposomes are comprised of phosphatidyl choline and cholesterol.

24. An MRI contrast agent of Claim 23 wherein said liposomes comprise about 60 to about 80 mole percent phosphatidyl choline and about 20 to about 40 mole percent cholesterol.

25. An MRI contrast agent of Claim 19 wherein said liposomes are selected from the size range consisting of about 20 to less than 50 nm.

26. An MRI contrast agent of Claim 25 wherein said liposome size range is about 20 to about 35 nm.

27. An MRI contrast agent of Claim 26 wherein said liposome size is about 35 nm.

28. An MRI contrast agent of Claim 19 wherein said liposomes encapsulate therein a paramagnetic agent.

29. An MRI contrast agent of Claim 28 wherein said paramagnetic agent is a lanthanide or transition element bound to a complexing agent.

30. An MRI contrast agent of Claim 29 wherein said lanthanide or transition elements are selected from the group consisting of Gd(III), Mn(II), Cu(II), Cr(III), Fe(II), Fe(III), Co(II), Er(II), Ni(II), Eu(III) and Dy(III).

31. An MRI contrast agent of Claim 29 wherein said complexing agent is selected from the group consisting of DTPA, EDTA, DOTA, DO3A, B-19036, HBED, DPDP, NOTA and TETA.

32. An MRI contrast agent of Claim 29 wherein said lanthanide element is Gd(III) and said complexing agent is DTPA.

33. An MRI contrast agent of Claim 19 which is a NMR contrast agent.

34. An MRI contrast agent of Claim 19 which is an MRI contrast agent for use in a liver or spleen.

35. A method for diagnosing the presence of tumorous tissue in a patient comprising (i) administering to the patient liposomes of Claim 19 and (ii) scanning the patient using MRI to obtain visible images of any tumorous tissue.

36. A method of Claim 35 wherein the MRI used is NMR.

37. A method of Claim 35 wherein the method is used to diagnose the presence of tumorous tissue in the liver and spleen of said patient.

38. A method of imaging the blood pool of a patient comprising (i) administering to the patient liposomes of Claim 19 and (ii) scanning the patient using MRI to obtain visual images of the location of the blood pool.

39. A method of Claim 38 wherein the MRI used is NMR.

40. A method of Claim 38 which is used to assess the vascularity of healthy or tumorous tissues in the patient.

41. A method of Claim 38 which is used to determine blood volume of the patient.

42. A method of Claim 38 wherein the method is used to assess vascular leakage in the patient.

FIGURE 2

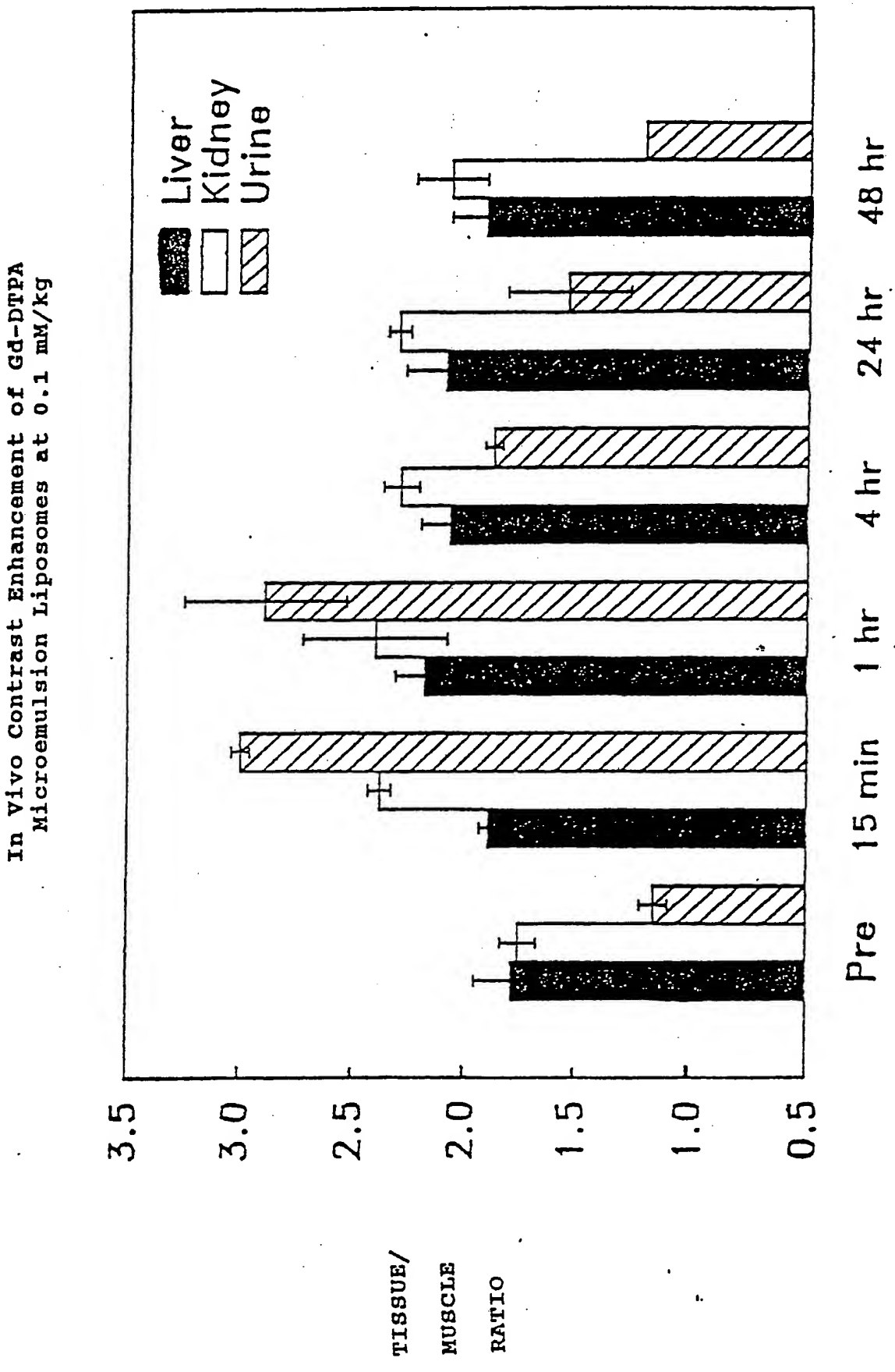

In Vivo Contrast Enhancement of Gd-DTPA
Microemulsion Liposomes at 0.1 mM/kg
Liver
Kidney
Urine
TISSUE/
MUSCLE
RATIO
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Pre
15 min
1 hr
4 hr
24 hr
48 hr

FIGURE 3

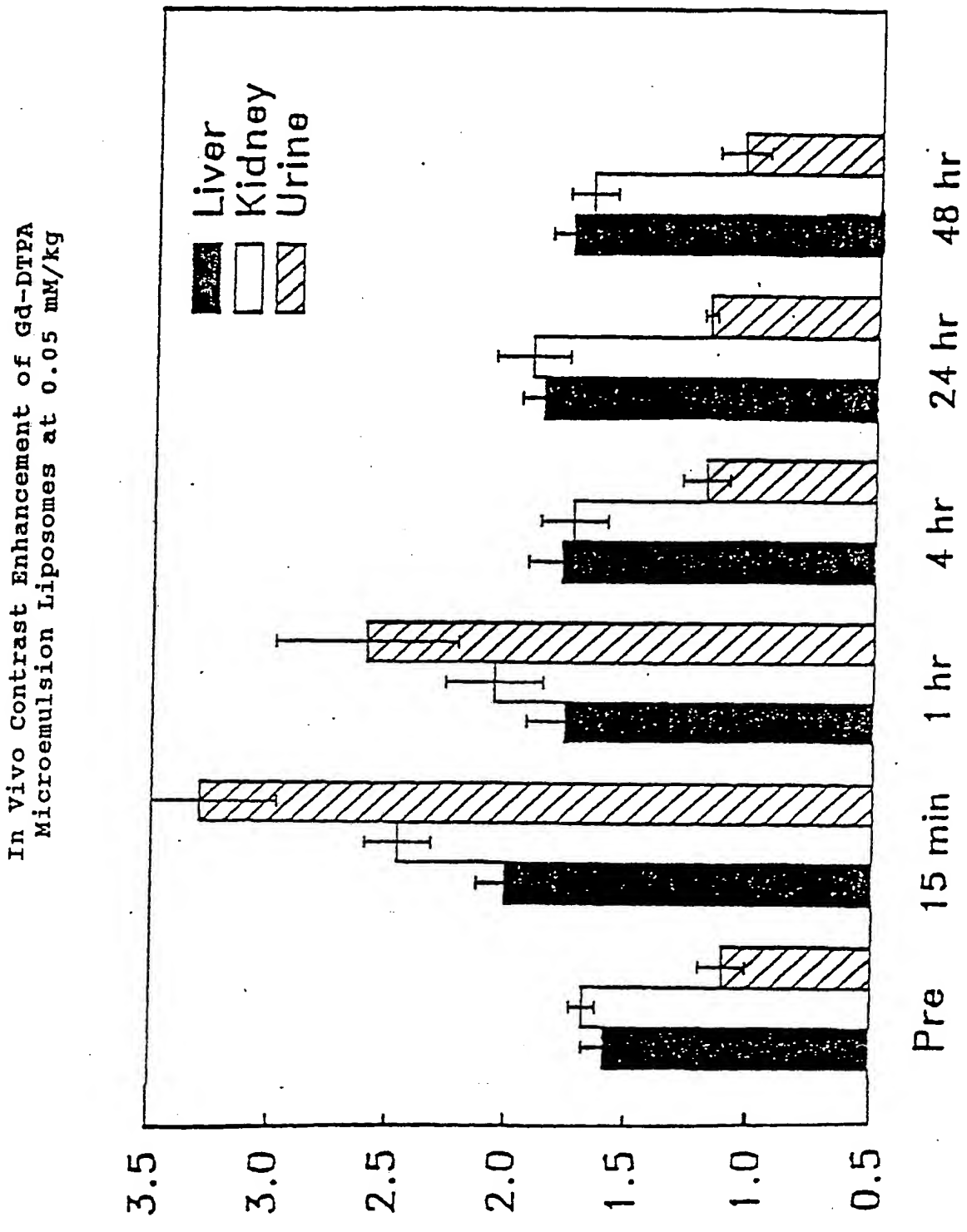

In Vivo Contrast Enhancement of Gd-DTPA
Microemulsion Liposomes at 0.05 mM/kg
Liver
Kidney
Urine
TISSUE/
MUSCLE
RATIO
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Pre
15 min
1 hr
4 hr
24 hr
48 hr

FIGURE 4

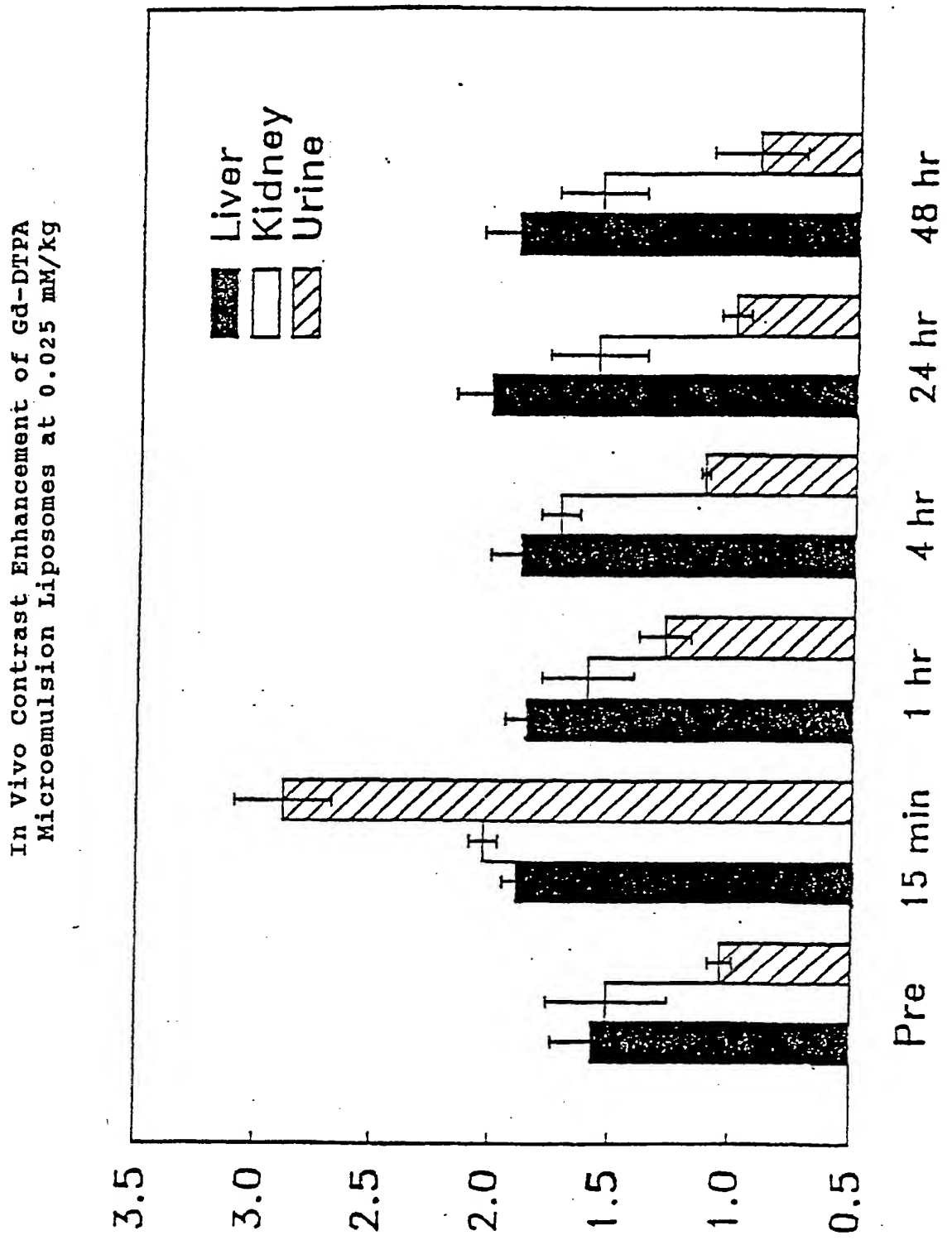
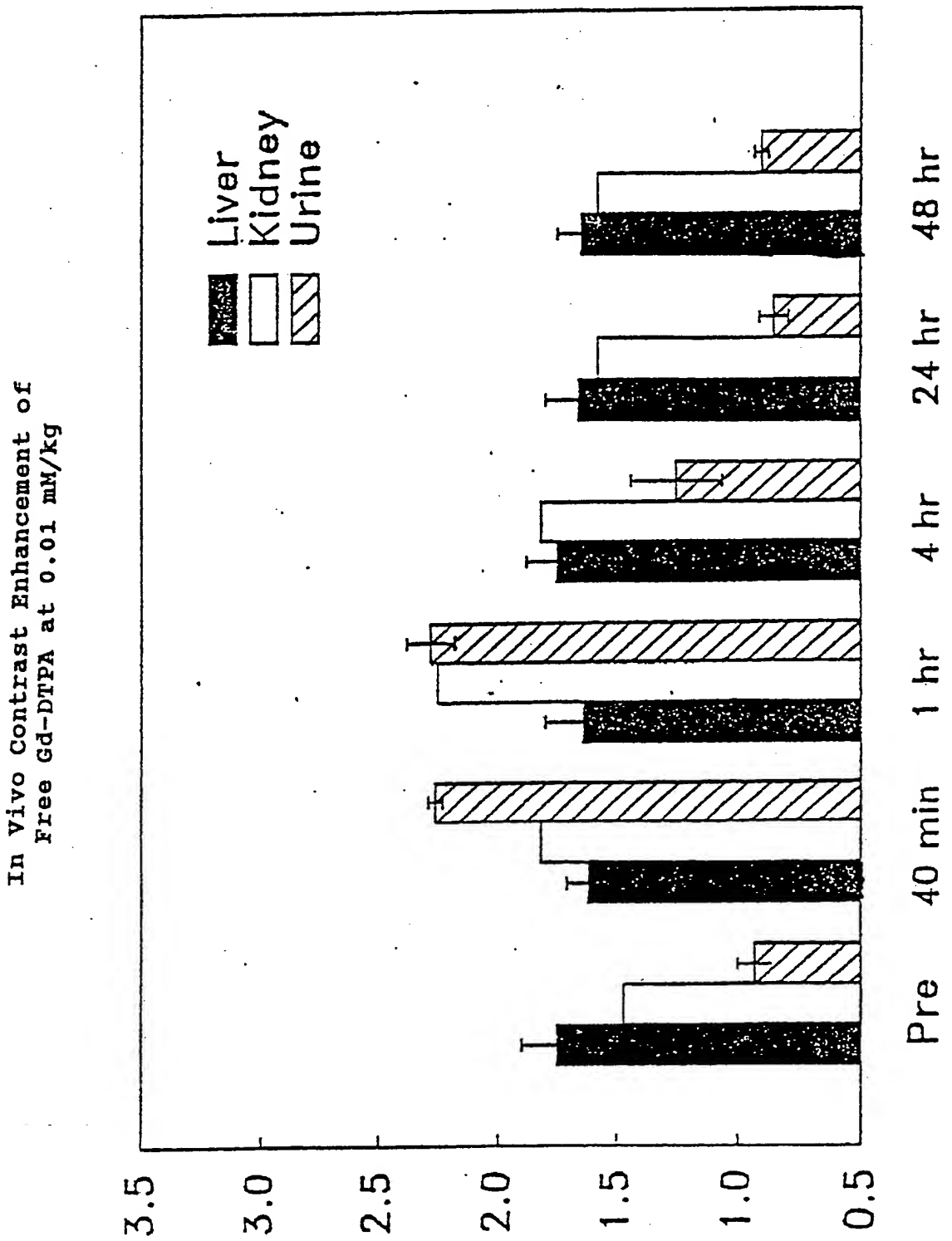

In Vivo Contrast Enhancement of Gd-DTPA
Microemulsion Liposomes at 0.025 mM/kg
Liver
Kidney
Urine
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Pre
15 min
1 hr
4 hr
24 hr
48 hr

FIGURE 5


In Vivo Contrast Enhancement of
Free Gd-DTPA at 0.01 mM/kg
Liver
Kidney
Urine
TISSUE/
MUSCLE
RATIO
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Pre
40 min
1 hr
4 hr
24 hr
48 hr

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05040

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61B 6/00;
U.S.Cl: 128/654; 424/1.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	128/653, 654, 659; 424/1.1, 4, 9, 450; 436/173, 829 264/4.3, 4.32; 428/402.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,675,173 (WIDDER) 23 JUNE 1987. See entire document.	1-42
Y	Biochimica et. Biophysica Acta, Vol. 858, 1986, MAYER ET AL., "Vesicles of Variable Sizes Produced by a Rapid Extrusion Procedure," pp. 161-168. See page 163 column 1 to page 167.	1-42
A	US,A, 4,192,859 (MACKANESS ET AL) 11 MARCH 1980. See abstract, column 5 lines 45-60.	1-42

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

02 February 1990

Date of Mailing of this International Search Report

01 MAR 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

Sharon E. Rose

Sharon E. Rose

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US,A, 4,532,089 (MAC DONALD), 30 JULY 1985. See abstract, column 1 line 40 to column 2 line 2.	1-42
A	Biochimica et Biophysica Acta, Vol. 812(1985) Hope et al, "Proudction of Large unilamellar Vesciles by a Rapid Extruision Procedure," pp. 55-65. See abstract.	1-42

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers 1, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	J. PHARM. PHARMACOL., VOL. 39 (1987) HIGGINS ET AL., "A Comparative Investigation of Glycinebetaine and DMSO as Liposome Cryoprotectives", pp. 577-582. See abstract, page 577, page 581.	1-42 -